In Variant Natural Killer T Cells Regulate Breast Cancer Response to Radiation and CTLA-4 Blockade

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Abstract

Purpose: Immunosuppressive mechanisms contribute major obstacles to the success of immunotherapy in cancer patients. We have shown that the combination of radiotherapy to the primary tumor and CTLA-4 blockade induces antitumor immunity, inhibiting metastases and extending the survival of mice bearing the poorly immunogenic and highly metastatic 4T1 mammary carcinoma. Similarly to patients with metastatic cancer, however, mice were seldom cured. Here we tested the hypothesis that invariant natural killer T (iNKT) cells, a subset with unique regulatory functions, can regulate the response to radiotherapy and CTLA-4 blockade.

Experimental Design: The growth of 4T1 primary tumors and lung metastases was compared in wild-type and iNKT cell–deficient (iNKT−/−) mice. Treatment was started on day 13 when the primary tumors were palpable. Mice received radiotherapy to the primary tumor in two doses of 12 Gy in combination or not with 9H10 monoclonal antibody against CTLA-4. Response to treatment was assessed by measuring primary tumor growth delay/regression, survival, and number of lung metastases.

Results: The response to radiotherapy plus 9H10 was markedly enhanced in the absence of iNKT cells, with 50% of iNKT−/− versus 0% of wild-type mice showing complete tumor regression, long-term survival, and resistance to a challenge with 4T1 cells. Administration of the iNKT cell activator α-galactosylceramide did not enhance the response of wild-type mice to radiotherapy plus 9H10. Tumor-infiltrating iNKT cells were markedly reduced in wild-type mice treated with radiotherapy plus 9H10.

Conclusions: iNKT cells play a major role in regulating the response to treatment with local radiotherapy and CTLA-4 blockade.

Preclinical models and clinical trials have provided the proof of principle that immunotherapy can treat cancer (reviewed in ref. 1). However, despite the development of multiple vaccination strategies to induce antitumor T cells, objective responses are seen only in a small fraction of patients, and it remains unclear what factors determine the success of any given treatment. Increased understanding of the complex networks of immune cells and cytokines that control immune system function has led to the identification of several regulatory and suppressive mechanisms as major obstacles to the success of immunotherapy (2). These are of two types: those that are tumor-induced and those preexisting in the host as the result of genetic predisposition, age, concurrent diseases, or previous therapies (3). Myeloid-derived suppressor cells (MDSC; ref. 4) belong to the first category because their accumulation in tumor-bearing mice and cancer patients is driven by tumor growth (5). Regulatory T cells play a key role in the maintenance of self-tolerance as well as tolerance to tumors (6). Natural regulatory T cells develop in the thymus and belong to the second category (7). In contrast, adaptive regulatory T cells are generated in the periphery from mature T cells, and their differentiation is induced by the tumor microenvironment (8, 9).

Another T cell subset with regulatory function, natural killer T (NKT) cells, has been implicated in both up- and down-regulation of immune responses (10). NKT cells have unique properties in that they can rapidly produce both Th1 and Th2 cytokines upon activation, and function as a powerful switch to turn on or off the innate and adaptive immune response in various diseases and conditions (10–12). NKT cells recognize glycolipid antigens presented by CD1d molecules, and in mice most express a canonical α-chain (Vα14Jα18) and are known as invariant NKT (iNKT) or type I NKT cells. In humans, the homologous population of iNKT cells expresses Vα24 (10). iNKT cells react with α-galactosylceramide, a
synthetic CD1d-binding glycolipid that has a strong agonistic activity, whereas the noninvariant or type II NKT cells, although restricted by CD1d, do not bind α-galactosylceramide (13).

In cancer, NKT cells have been associated with spontaneous immunosurveillance in some experimental systems (14, 15), but have been shown to suppress immunosurveillance in others (16, 17). Recent work by Terabe et al. (18), suggests a functional difference between the two subsets of NKT cells in the regulation of antitumor immunity, with type II NKT cells being mostly responsible for immunosuppression. An immunoregulatory pathway that links the production of interleukin (IL)-13 by NKT cells to the induction of transforming growth factor β (TGFβ) production by MDSC has been shown to be responsible for the inhibition of antitumor CTL that results in tumor recurrence in some tumor models (16, 19, 20) but not in others (21, 22). Overall, the mechanisms of NKT cell–mediated immunoregulation in cancer involve multiple pathways and remain largely undefined.

Activation of iNKT cells by systemic administration of α-galactosylceramide to mice and humans has been shown to induce the rapid production of IFN-γ and IL-4 and other Th1 and Th2 cytokines (10). Importantly, in several preclinical cancer models this resulted in effective antitumor immunity mediated by the downstream activation of antigen-presenting cells, and NK and CD8-positive T cells (23). In initial clinical trials, however, α-galactosylceramide has not shown significant anticancer activity, possibly due to partially impaired NKT cell function in cancer patients (24).

Accumulated evidence in experimental models supports the development of a novel therapeutic approach based on the combination of ionizing radiation therapy with immunotherapy for the treatment of cancer (reviewed in refs. 25, 26). In most cases clinical trials testing the efficacy of novel treatments are done in patients with advanced-stage disease, a population for which the need for new therapies is most urgent, but that also presents with an immunologic environment largely altered by the tumor. To mimic this situation, we have employed as a model the poorly immunogenic 4T1 mouse mammary carcinoma. After s.c. inoculation 4T1 cells grow to form a highly invasive primary tumor that early on sheds spontaneous metastases to the lungs and other organs (27). Mice usually die of metastatic disease to the lungs. We previously tested the combination of radiotherapy with CTL-associated protein 4 (CTLA-4) blockade approximately two weeks after implantation in mice, when primary tumors are palpable and metastatic cells have already spread systemically (28). Whereas single modality treatment was ineffective, the combination of local radiotherapy to the primary tumor and CTLA-4 blockade elicited CD8 T-cell–dependent antitumor immunity. The immune response effectively inhibited the growth of spontaneous lung metastases, prolonging the survival time of animals. Cure was rare, however, and most mice eventually succumbed to their disease (28).

In this study we investigated whether the disruption of immunoregulatory circuits can improve the response to treatment with radiotherapy and CTLA-4 blockade. Our data indicate that whereas 4T1 tumors grew equally well in wild-type mice and mice lacking iNKT cells (iNKT-/-), the latter developed a CD8 response that partially inhibited metastases but not primary tumor growth. This observation is in agreement with a minor role of iNKT cells in regulating spontaneous immunosurveillance in the 4T1 model (18). Remarkably, as compared with wild-type mice, iNKT-/- mice showed a marked improvement in survival and cure rate following treatment with radiotherapy and CTLA-4 blockade, suggesting that iNKT cells can play a major role in regulating the response to treatment. Administration of α-galactosylceramide did not improve the response of wild-type mice to radiotherapy and CTLA-4 blockade. Despite the differential response to treatment, wild-type and iNKT-/- mice showed a similar systemic and intratumoral increase in MDSC around the time treatment was started, suggesting that the degree of MDSC accumulation does not predict the response to treatment.

### Materials and Methods

**Mice.** Six- to eight-week-old BALB/c mice were obtained from Taconic Animal Laboratory. iNKT-/- (Vα14 Jα18-deficient) mice (29) in the BALB/c background obtained from M. Taniguchi (RIKEN Research Center for Allergy and Immunology) were bred at New York University and used between 6 and 12 wk of age. All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

**Cells and reagents.** 4T1 is a BALB/c mouse–derived mammary carcinoma cell line (provided by Fred Miller, the Michigan Cancer Center; ref. 27) and A20 is a BALB/C mouse–derived B-cell leukemia/lymphoma (30). 4T1 cells were grown in DMEM (Invitrogen Corporation) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 × 10–5 mol/L 2-mercaptoethanol, and 10% fetal bovine serum (Gemini Bio-Products; complete medium). These cells were found to be free of contamination by mycoplasma by the Mycoplasma detection kit (Roche Diagnostics). Anti-CTLA-4 hamster monoclonal antibody (mAb) 9H10 was purified as previously described (28). Control hamster IgG was purchased from Jackson ImmunoResearch Laboratories. Purified anti-CD4 (GK1.5), anti-CD8 (2.43) rat mAb and control rat IgG were purchased from BioExpress, Inc.
Tumor challenge and treatment. The mice were injected s.c. in the right flank with 5 × 10^4 4T1 cells in 0.1 mL of DMEM without additives on day 0. Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes were calculated as length × width^2 × 0.52. On day 13, when tumors reached the average diameter of 5 mm (approximately 65 mm^3 in volume) animals were randomly assigned to various treatment groups, as indicated. Radiotherapy was administered as previously described (28). Briefly, all mice (including mice receiving mock radiation) were lightly anesthetized by i.p. injection of averatin (240 mg/kg) then positioned on a dedicated plexiglass tray, and the whole body was protected by lead shielding, except for the area of the tumor to be irradiated. Radiotherapy was delivered to a field including the tumor with 5-mm margins using a gamma CO radiation source by two fractions of 12 Gy each on days 13 and 14. Control hamster IgG and 9H10 were given i.p. at 200 μg at 1, 4, and 7 days after radiotherapy. α-Galactosylceramide (Alexis Biochemicals) was dissolved in DMSO and diluted in PBS supplemented with 0.5% polysorbate-20 (w/v) prior to injection and given i.p. at 100 ng/mouse twice a week, starting on day 1 postradiotherapy. Tumor growth was evaluated every 2 to 3 d until death or sacrifice when tumor dimensions exceeded 5% body weight or mice showed dyspnea, abnormal posture, >20% body weight loss, difficulty with ambulation, or any other clinical sign of metastatic disease causing significant pain or distress, according to institutional guidelines. In some experiments, mice that rejected the tumors and survived tumor-free until day 120 were injected in the controlateral flank with a tumorigenic inoculum of either 4T1 or A20 cells and followed for up to 60 d for tumor development.

Clonogenic lung metastases assay. For determination of the number of clonogenic lung metastases, lungs were harvested on day 33 or 35 post-s.c. injection in the right flank with 5 × 10^4 4T1 cells and processed as previously described (27). Briefly, lungs from each individual animal were minced into 1-mm pieces, and digested with 5 mL enzyme cocktail containing 1 mg/mL collagenase IV and 6 units/mL elastase, both from Sigma Chemical Company, in PBS for 1 h at 4°C with rotation. Cell suspensions were filtered through 70-μm nylon cell strainers and washed two times with HBSS, then resuspended in complete medium. Serial 3- to 5-fold dilutions were plated in 10-cm tissue culture dishes in the presence of 60 μl/mL 6-thioguanine (2-amino-6-mercaptopurine; Sigma Chemical Company) to allow only the growth of 4T1 cells which are resistant to this drug (27). When colonies of growing 4T1 cells became visible (8-14 d) the plates were washed with PBS, fixed with methanol, and stained with Crystal violet. The colonies were counted independently by two to three individuals, blinded to the group to which each mouse belonged, and the total number/lungs was calculated for each animal.

In vivo T-cell subset depletion. Depletion of CD4 and CD8 T-cell subsets was done by injecting GK1.5 or 2.43 mAb i.p. at 100 μg/mouse on 3 consecutive d, starting 1 d before the s.c. injection in the right flank with 5 × 10^4 4T1 cells. The depletion was maintained by repeated weekly injections of mAb. Depletion was confirmed by testing spleen cells from control animals for the presence of CD4 and CD8 T-cells using non–cross-reactive FITC-RMA-44 and PE-anti-CD8β mAb (BD Pharmingen).

Mice vaccination. Wild-type and iNKT-/- mice were vaccinated with 10^6 irradiated (100 Gy) 4T1 cells s.c. in the right flank 3 times at weekly intervals. Control mice received DMEM. Seven days after the last vaccination the mice were challenged with a tumorigenic inoculum (5 × 10^4) of 4T1 cells s.c. in the opposite flank and followed for tumor development.

Analysis of MDSC and iNKT cells infiltrating 4T1 tumors. The tumors were dissected carefully removing all surrounding normal tissue, minced into 1-mm pieces, and digested with collagenase D (400 U/mL) for 25 min at 37°C in a shaker. Ten milliliters of the enzyme solution were used for every gram of tumor tissue. Obtained cell suspensions were filtered through 40-μm nylon cell strainers and washed two times with HBSS. Aliquots of 10^6 tumor-derived cells were incubated with antimouse CD11b/PE (BD Pharmingen) to identify MDSC, or with PE-conjugated mCD1d/PBS7 tetramer (provided by the NIH Tetramer Facility, ref. 31) and FITC-conjugated CD3 to identify iNKT cells. Unloaded PE-conjugated mCD1d tetramer was used as control. Samples were analyzed using a FACScan flow cytometer and Flowjo version 6.4.3 (Tree Star).

Measurement of TGF β1 production. 1 × 10^7 spleen cells from individual mice were cultured on a 1% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 24-well plate. Supernatants were harvested and stored at -80°C. The concentration of TGF β1 was determined in duplicate samples using the QuantiKine TGF β1 Immunoassay Kit (R&D Systems) following the manufacturer’s instructions. Background readings for culture medium were subtracted from all samples. As previously reported (19), acidification of the samples was required to detect the latent form of TGF β1 produced in vitro by MDSC.

Immunostaining of tumor sections. 4T1 tumors from treated and untreated wild-type and iNKT-/- mice were harvested on day 29 posttumor inoculation, fixed for 1 h at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in optimum cutting temperature medium. Sections (8 μm) were incubated with 0.1% Tween-20 and 0.01% Triton-X100 for 20 min, followed by 4% rat serum in 4% bovine serum albumin/PBS for an additional 30 min. Sections were stained with PE-Texas-Red-conjugated rat antimouse CD4 or PE-conjugated rat antimouse CD8αa (Caltag), and counterstained with 5 μg/mL 4',6-diamidino-2-phenylindole (Sigma). Images were obtained using a Nikon Eclipse 800 deconvolution microscope. The CD4 and CD8 T cells were counted in three randomly selected (20×) fields in each tumor.

Statistical analysis. ANOVA based on ranks was used to assess differences among animal groups defined by genotype and/or treatment received with respect to tumor weight, tumor volume, or the number of lung metastases at a fixed time point. Specifically, each end point (tumor weight, tumor volume, number of metastases) was first converted to ranks within each experiment and the ranks were then used as the dependent variable in the analysis of variance. Ranks were used in place of the observed values to better satisfy underlying distributional assumptions. When the treatment groups constituted a × 2 factorial design (presence/absence of CTLA-4 blockade/radiotherapy), the analysis examined the main effects for each treatment modality (radiotherapy, CTLA-4 blockade) and the interaction between the modalities. The log-rank test was used to compare animal groups in terms of overall survival, defined as time to death or sacrifice. The median and mean survival times within each treatment arm were estimated using the Kaplan-Meier product-limit method, and a 95% confidence interval for each median survival time was derived on the basis of a sign test. All reported P values are two-sided and were declared statistically significant at the 5% level. The statistical computations were carried out using SAS for Windows, version 9.0 (SAS Institute).

Results

iNKT-/- mice develop a spontaneous CD8-dependent antitumor immune response inhibiting metastases. To investigate the role of the host immune status in tumor growth and response to treatment we injected mice lacking iNKT cells with 4T1 cancer cells. Whereas primary tumors grew at the same rate in iNKT-/- and wild-type mice (not shown) and primary tumor weight was not significantly different on day 34 (mean ± SD, 1,364 ± 895 and 873 ± 234 mg for iNKT-/- and wild-type mice, respectively; P = 0.41), the number of metastatic cells in the lungs was significantly lower in iNKT-/- mice (mean ± SD, 525.4 ± 1461.9 and1,364.0 ± 895.5 for iNKT-/- and wild-type, respectively; P = 0.0009; Fig. 1A). The depletion of CD8 T cells or CD4 and CD8 T cells abrogated the difference in lung
metastases between wild-type and iNKT-/− mice, whereas the depletion of CD4 T cells did not have any effect, indicating that CD8 T cells are required for the inhibition of lung metastases observed in iNKT-/- mice (Fig. 1B). Interestingly, primary tumor growth was not affected by depletion of CD8 or CD4 T cells but a statistically significant increase in tumor weight \((P < 0.01)\) was seen in double-depleted mice (Fig. 1C).

The spontaneous development of antitumor CD8 T cells could be due to increased intrinsic immunogenicity of 4T1 cells in iNKT-/− mice. To test this hypothesis, wild-type and iNKT-/− mice were immunized repeatedly with 4T1 cells inactivated by irradiation, followed 7 days after the last immunization by challenge with \(5 \times 10^4\) live 4T1 cells. If antitumor CD8 cells had developed following vaccination, they would prevent or inhibit growth of the early small tumors. In contrast, all wild-type and iNKT-/− mice developed tumors, and there was no significant difference in tumor weight between 4T1-vaccinated and nonvaccinated mice (Fig. 1D). Therefore, 4T1 cells do not show increased immunogenicity in iNKT-/− mice.

In the absence of iNKT cells, mice bearing established 4T1 mammary carcinoma show a markedly enhanced therapeutic response to treatment with local radiotherapy and CTLA-4 blockade. We have shown that wild-type mice bearing the poorly immunogenic 4T1 mammary carcinoma develop antitumor CD8 T cells inhibiting spontaneous lung metastases and extending their survival when treated with the combination of local radiotherapy to the primary tumor and CTLA-4–blocking mAb 9H10, whereas each single modality did not have an effect on survival (28). However, complete cure of mice with well-established disease remained rare: 0% to 15% of the mice were cured in different experiments (28). This suggested that the duration and/or potency of the antitumor immune response elicited by treatment was limited in most animals. To test if iNKT cells play a role in response to treatment, iNKT-/− mice with established 4T1 tumors were randomly assigned to be treated with radiotherapy, 

4 Our unpublished results.
CTLA-4 blockade, or a combination of the two modalities. In the absence of treatment, tumors grew progressively, and all mice were dead by day 49. Radiotherapy alone was able to cause a significant ($P < 0.05$) growth delay of the irradiated tumor, and complete regression in 2 of 9 mice. Despite the fact that the growth of all irradiated tumors was delayed, there was no statistically significant improvement in survival ($P = 0.16$; Fig. 2A and B). This is consistent with the fact that radiotherapy as a single modality cannot significantly inhibit the lung metastases outside of the field of radiation and, therefore, cannot extend survival, as previously observed in wild-type mice (28). However, the two iNKT-/- mice treated with radiotherapy alone that showed complete regression of the primary tumor remained tumor-free on day 120, and showed the development of a protective antitumor response, as described below.

iNKT-/- mice receiving CTLA-4–blocking mAb as a single modality showed a significant ($P < 0.05$ from day 21 compared with control), although less pronounced than that obtained with radiotherapy, tumor growth delay, and the tumor regressed completely in 1 of 9 mice (Fig. 2A). This animal was cured of tumor, whereas the rest of the group showed a significant ($P = 0.002$) but relatively modest increase in survival when compared with the control group (Fig. 2B). This differs from results obtained in wild-type mice in which CTLA-4 blockade did not have any effect on primary tumor growth or survival (28).

Treatment with the combination of radiotherapy and CTLA-4 blockade caused complete regression of the irradiated tumor in 6 of 9 mice (Fig. 2A and B), and a marked extension of their survival with all animals alive on day 74 ($P < 0.0001$ compared with control group). All tumor-free mice that survived until day 120 were considered “cured” and were rechallenged with a tumorigenic inoculum of 4T1 cells (two mice in radiotherapy, one in 9H10, and three in radiotherapy plus 9H10 group) or the syngeneic but unrelated A20 lymphoma cells (three mice in radiotherapy plus 9H10 group). All 6 mice rejected the 4T1 tumor, whereas 5 of 5 naïve mice challenged at the same time developed tumors. In contrast, the A20 tumor grew in the three survivor mice and three naïve mice (data not shown). These data indicate that iNKT-/- mice cured of tumor developed a long-lasting tumor-specific memory response.

To directly determine the effect of treatment on lung metastases, another experiment was done in which tumorbearing mice were treated as above and lungs analyzed on day 35. Although single treatment with either radiotherapy or 9H110 showed a tendency to lower the number of metastatic cells, the difference was not statistically significant compared with control IgG-treated mice (Fig. 2C). In contrast, mice treated with radiotherapy and CTLA-4 blockade showed complete inhibition of lung metastases (Fig. 2C). The observation that the effect of radiotherapy on lung metastases was significant only in the presence of 9H110 is consistent with a synergism between radiotherapy and CTLA-4 blockade.

Next, responses to the combination treatment with radiotherapy and CTLA-4 blockade were directly compared between 4T1 tumor–bearing wild-type and iNKT-/- mice. In the absence of treatment, there was no difference in primary tumor growth between wild-type and iNKT-/- mice (Fig. 3A). However, untreated iNKT-/- mice showed a small but statistically significant increase in survival ($P = 0.008$; mean, 43.8 and 37.1 days for iNKT-/- and wild-type mice, respectively). This is consistent with the CD8-mediated inhibition of metastases in iNKT-/- mice (Fig. 1).

Treatment with radiotherapy and CTLA-4 blockade initially caused a significant primary tumor growth delay in both wild-type and iNKT-/- mice, but only iNKT-/- mice went on to
achieve complete tumor regression and cure, as shown by the absence of tumor on day 150 in 50% of the animals (Fig. 3A and B). None of the wild-type mice survived long-term, suggesting that only in iNKT-/- mice was the duration and potency of the antitumor immune response sufficient to completely eradicate the tumor.

Analysis of tumor-infiltrating lymphocytes on day 29 showed that CD4-positive cells were present in similar quantities in 4T1 tumors growing in wild-type and iNKT-/- mice, and were not significantly increased by treatment with radiotherapy and CTLA-4 blockade (Fig. 3C). In contrast, following treatment there was a significant increase in the number of CD8-positive cells infiltrating 4T1 tumors in both wild-type and iNKT-/- mice \( (P < 0.01) \). Importantly, the number of CD8-positive tumor-infiltrating lymphocytes was higher in iNKT-/- mice than in wild-type mice, and this difference was highly significant following treatment \( (P = 0.006) \).

The iNKT cell-specific activator \( \alpha \)-galactosylceramide does not improve the response of tumor-bearing wild-type mice to treatment with radiotherapy and CTLA-4 blockade. \( \alpha \)-Galactosylceramide, a potent activator of iNKT cells, has been shown to induce powerful antitumor immune responses in several tumor models (23). Moreover, in a burn injury model, administration...
of α-galactosylceramide was able to prevent the immunosuppression that is mediated by iNKT cells (33). To determine whether administration of α-galactosylceramide to 4T1 tumor-bearing mice could activate iNKT cells to carry out stimulatory rather than inhibitory functions, and improve the response of wild-type mice to radiotherapy and CTLA-4 blockade, mice were given α-galactosylceramide starting one day postradiotherapy. As a single modality α-galactosylceramide did not have any effect on tumor growth, and it did not improve the tumor growth delay or survival caused by treatment with radiotherapy and radiotherapy plus 9H10 (Fig. 4 and data not shown). However, a modest but statistically significant tumor growth delay was seen in mice treated with α-galactosylceramide and 9H10 (P = 0.0396 compared with control vehicle–treated mice; Fig. 4).

Overall, these data indicate that administration of α-galactosylceramide to wild-type mice with established tumors does not improve their response to treatment with radiotherapy and CTLA-4 blockade.

Reduction in tumor-infiltrating iNKT cells after treatment with radiotherapy and CTLA-4 blockade. The presence within tumors of T cells with regulatory function has been shown to play an important role in the suppression of antitumor immunity (8). To determine whether iNKT cells were present within 4T1 tumors growing in wild-type mice, cell suspensions prepared from 4T1 tumors and lungs were stained with CD3 and CD1d tetramers loaded with the α-galactosylceramide analog PBS-57 (31). In untreated mice iNKT cells represented ~4% of T cells infiltrating 4T1 tumors and 3% of T cells isolated from the lungs (Fig. 5A and B). Importantly, there was a pronounced decline in iNKT cells in both primary tumor and lungs following treatment with radiotherapy plus 9H10 (Fig. 5B and C). Overall, these data indicate that iNKT cells are recruited to “primary” 4T1 tumors and their metastases, and that a treatment stimulating antitumor immunity leads to a relative decline in iNKT cell numbers, an observation that supports a regulatory role for iNKT cells.

Wild-type and iNKT-/- mice do not differ in the tumor-driven expansion and recruitment of MDSC to tumors. Suppression of antitumor immune responses by NK cells has been linked to their ability to induce TGFβ production by MDSC (19). To determine whether the markedly increased response to treatment in iNKT-/- mice was due to decreased accumulation of MDSC, the numbers of MDSC in the spleen and primary tumors of wild-type and iNKT-/- mice were analyzed on day 14 of tumor growth, corresponding approximately to the time therapy was initiated. MDSC accumulation in spleens as well as primary tumors was similar between wild-type and iNKT-/- mice (Fig. 6A and B). Next, spleen cells of tumor-bearing wild-type and iNKT-/- mice were tested for secretion of TGFβ in short-term cultures. As previously reported for the 15-12RM tumor model (19), the secretion of TGFβ by spleen cells from 4T1 tumor-bearing wild-type mice was significantly increased compared with healthy mice (P < 0.05). This was true also for cells derived from iNKT-/- mice (Fig. 6C). Although the baseline production of TGFβ did not differ significantly between wild-type and iNKT-/- healthy mice (P = 0.35), tumor-bearing iNKT-/- mice produced more TGFβ than tumor-bearing wild-type mice, indicating that the production of TGFβ was not dependent on the presence of iNKT cells. Overall, these results are consistent with a recent report showing that NK cells are not required to activate MDSC (34). They also indicate that the improved response to treatment with radiotherapy and CTLA-4 blockade seen in tumor-bearing iNKT-/- mice cannot be explained by differences in MDSC numbers or functional activation to produce TGFβ.

Discussion

In this study, we show that mice lacking the iNKT cell subset and bearing well-established 4T1 tumors respond to treatment with the combination of local radiotherapy and CTLA-4 blockade with markedly increased overall survival and cure rate as compared with wild-type mice (Figs. 2 and 3 and ref. 28). The improved response to treatment was not a result of increased immunogenicity of 4T1 cells in iNKT-/- mice because the growth of 4T1 tumors was similar in wild-type and iNKT-/- mice and vaccination with irradiated tumor cells did not induce...
a protective antitumor response (Fig. 1D). However, iNKT-/- mice developed a spontaneous CD8 response inhibiting lung metastases (Fig. 1).

The development of an antitumor immune response that is at least partially effective against metastases in face of a poorly immunogenic growing primary tumor is an example of concomitant tumor immunity (35). This well-known phenomenon is consistent with the notion that invasive cancers cause tissue damage and the generation of inflammatory signals attracting innate immune cells and eventually leading to the activation of the adaptive immune system (36). Regulatory T cells have been shown to suppress concomitant immunity to the poorly immunogenic B16 melanoma (37). Our data suggest that iNKT cells play a similar role in the case of the poorly immunogenic 4T1 carcinoma and are consistent with a previous observation that iNKT-/- mice have improved immunosurveillance against 4T1 metastases compared with wild-type mice (18). Importantly, whereas Terabi et al. (18) detected the immune response against metastases after surgical removal of the primary tumor, our work provides the evidence that it is generated in the absence of any surgical manipulation that could potentially contribute to the development of the antitumor response (38).

Treatment of iNKT-/- mice with local radiotherapy used as a single modality caused a reduction in lung metastases that, however, was not statistically significant (Fig. 2C). Consistent with this, despite the complete "cure" obtained in ~20% of the mice, the effect of radiotherapy alone on survival was not significant \( (P = 0.16; \text{Fig. 1B}) \). This suggests that radiotherapy by itself was either not sufficient to induce a T cell response against this poorly immunogenic tumor, or that if induced it was suppressed (39). However, in combination with CTLA-4 blockade there was a highly significant antitumor effect leading to complete clearance of primary tumor and metastatic disease in over half of the mice (Figs. 2 and 3). Overall, these data are consistent with the hypothesis that radiotherapy causes changes in the tumor microenvironment that can promote the afferent and efferent phases of the antitumor immune response (26, 32, 40).

CTLA-4 blockade used as a single modality had a modest but detectable effect in inhibiting tumor growth and extending the survival of iNKT-/- but not wild-type mice (Fig. 2 and ref. 28). This result was confirmed in two additional experiments (data not shown). CTLA-4 blockade used as a single treatment is known to be effective only against more immunogenic tumors in wild-type mice (41). This suggests that besides the intrinsic immunogenicity of a tumor, preexisting host-specific factors can determine the response to CTLA-4 blockade. It is intriguing to consider whether the number or activity of iNKT cells could be a determinant of therapeutic responses mediated by anti-CTLA-4 antibodies in cancer patients (42).

Immunosuppression by NKT cells has been previously shown to be mediated by MDSC and TGF\( \beta \) in some tumor models (16, 19, 20). In the 4T1 model, the increase in splenic MDSC in tumor-bearing mice was shown to correlate with the suppression of the immune response against metastases (43). Given the development of a spontaneous CD8 response inhibiting metastases in iNKT-/- mice, we expected to find less MDSC in iNKT-/- than in wild-type mice. In contrast, neither MDSC nor TGF\( \beta \) production was reduced in the spleen of iNKT-/- mice (Fig. 6). This suggests that MDSC likely exert their suppression locally, within the growing primary tumors, as previously shown by Kusmartsev et al. (44). In mice treated with radiotherapy, sensitization of CD11b-positive MDSC by tumor-derived antigens that are released by radiation (40) could promote their destruction by antitumor CTL activated by anti-CTLA-4 antibodies leading to complete regression of established primary tumors.

Administration of the potent iNKT cell activator α-galactosylceramide did not improve the response of wild-type mice to
treatment with local radiotherapy and CTLA-4 blockade (Fig. 4). In a recent report, 4T1 tumor–bearing mice given α-galactosylceramide in a similar dose/schedule (100 ng every 4 days) in combination with anti-DR5 mAb to induce tumor cell apoptosis and anti-4-1BB mAb to provide costimulatory signals to T cells, showed significant rates of tumor rejection (45). This suggests that iNKT cell immunostimulatory function can be “rescued” by activation with α-galactosylceramide, but the outcome of this activation is influenced by the immunologic environment in which this takes place. Indeed, α-galactosylceramide has been shown to promote the regulatory functions of iNKT cells when given to mice with autoimmune diseases (11). Therefore, it will be important to determine which immunotherapy strategies may be successfully combined with α-galactosylceramide to elicit antitumor immune responses in cancer patients.

It has been proposed that there is a dichotomy between the two main subsets of NKT cells, noninvariant (or type II) NKT cells being sufficient for negative regulation of the antitumor immune response and iNKT cells being mostly responsible for protection (18). Our study shows that the selective absence of iNKT cells is sufficient to dramatically enhance the response to treatment with radiotherapy and CTLA-4 blockade in mice with an established metastatic mammary carcinoma. Importantly, at least in this tumor model, an antitumor immune response was detectable even in the absence of treatment, consistent with a role for iNKT cells in the suppression of antitumor immunity. Therefore, it is likely that in cancer, similarly to other autoimmune/inflammatory diseases (10), the regulatory role of invariant and noninvariant NKT cells is not absolute, but is influenced by both the immunologic environment of the tissue/tumor and unknown factors of the host. For instance, a glycolipid produced by melanoma cells has been shown to be cross-presented by dendritic cells (DC) and to induce production of IL-10 by NKT cells (46). The presence of iNKT cells in 4T1 tumors (Fig. 5) raises the question of whether they may recognize a tumor-derived glycolipid. Few endogenous CD1d ligands are currently known. Identification of CD1d ligands that are expressed by cancer cells and may be recognized by different NKT cell subsets and/or with different affinity will help clarify the role of NKT cells in regulation of antitumor immunity (47).

Previous studies have shown a marked enhancement of antitumor immunity in CD1d-deficient mice that lack both subsets of NKT cells (18, 21, 22), and these results have been interpreted as evidence of a strong regulatory role of type II NKT cells. However, the recent evidence that some mouse tumors express low levels of CD1d (48) raises the possibility that CD1d may act as a neoantigen in CD1d-deficient mice, similarly to STAT6 in STAT6-deficient mice (49). Although CD1d expression in 4T1 cells has not been detected by immunostaining and flow cytometry (ref. 18 and data not shown), using a more sensitive technique, real time reverse transcription-PCR, we have found that 4T1 cells express CD1d (Supplementary Fig. S1). Because CD1d-reactive T cells may be present in the

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**Fig. 6.** Accumulation of MDSC and TGFβ production in wild-type and iNKT-/- mice bearing 4T1 tumors. Wild-type and iNKT-/- mice were inoculated s.c. with 5 × 10⁴ 4T1 cells on day 0 (n = 5/group). Three non–tumor-bearing mice of each genotype served as controls. On day 14 primary tumors and spleens were harvested and obtained cell suspensions were stained with PE-anti-CD11b and Cy-anti-Gr-1 mAbs to detect MDSC. A, representative histograms of spleen from one healthy (top row) and one tumor-bearing (middle row) mouse showing the CD11b-positive Gr1-positive MDSC. Histograms from pooled tumors isolated from 5 wild-type and 5 iNKT-/- mice showing the CD11b-positive Gr1-positive MDSC (bottom row). Numbers indicate the percentage of MDSC. B, percentage of spleen MDSC in relation to the tumor weight in wild-type (closed triangles) and iNKT-/- (open circles) mice. Each symbol represents an individual mouse. For control healthy mice, one symbol representing the mean of 3 animals is shown. C, splenocytes isolated from tumor-free (−; n = 3) and tumor-bearing (+; n = 5) mice were cultured o.n. and the concentration of TGFβ in the culture supernatants was determined by ELISA. Data are the mean ± SE. In both wild-type and iNKT-/- mice spleen cells derived from tumor-bearing mice produced significantly more TGFβ than spleen cells derived from healthy mice (P < 0.05). Although the baseline production of TGFβ did not differ significantly between wild-type and iNKT-/- healthy mice (P = 0.35), tumor-bearing iNKT-/- mice produced more TGFβ than tumor-bearing wild-type mice (P < 0.01).
In conclusion, we show that mice developing a detectable repertoire of CD1d-deficient mice, their possible role in 4T1 tumor rejection will need to be addressed.

At the level of the intracellular tumor immunogenicity has often been invoked to explain these responses, focusing on the “immunologic” make-up of the host may be important for an improved understanding of the determinants of response to immunotherapy.

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References
